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E-mail: jzus@zju.edu.cn

Correspondence:

Full genome analysis of swine genotype 3 hepatitis E virus isolated from eastern China*

Jiang-bing SHUAI[1], Lu-huan LI[2], Ai-yun LI[3], Yong-qiang HE[1], Xiao-feng ZHANG[†‡1]

([1]*Zhejiang Academy of Science and Technology for Inspection and Quarantine, Hangzhou 310016, China*)

([2]*Department of Cardiopulmonary Rehabilitation, Hangzhou 128 Hospital, Hangzhou 310007, China*)

([3]*Women's Hospital, School of Medicine, Zhejiang University, Hangzhou 310006, China*)

[†]E-mail: zxf@zju.edu.cn

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Hepatitis E is believed to occur in both endemic and sporadic forms in developing countries, which causes a major public health problem in Asia and Africa (Meng, 2010; Wang *et al*., 2016a). Recent studies have documented that the disease is also endemic in many industrialized countries (Wenzel *et al*., 2011). The causative agent, hepatitis E virus (HEV), belonging to the genus *Orthohepevirus*, is a non-enveloped RNA virus with a single-stranded, positive-sense genome of approximately 7.2 kb (Smith *et al*., 2014). The genome consists of a short 5' un-translated region (UTR), three open reading frames (ORFs), and a 3' UTR containing a poly(A) tail (Meng, 2011). Four recognized major genotypes of HEV are identified: genotype 1 (Asian and African strains), genotype 2 (a Mexican strain), genotype 3 (primarily from America and Europe, and some Asian countries), and genotype 4 (mainly Asian strains) (Smith *et al*., 2016). Previous study revealed that HEV genotype 4 is the dominant zoonotic HEV genotype in China (Wang *et al*., 2016a). However, infections with HEV 3 have been found more commonly in recent years in China (Liu *et al*., 2012; Zhang *et al*., 2013). To date, only one full genome of Chinese swine genotype 3 HEV strain from Shanghai has been documented (Si *et al*., 2009). We report here the first full genome sequence of a genotype 3 swine HEV strain from Zhejiang, China.

Two hundred and seventy-three fecal samples were collected and suspended in 0.01 mol/L phosphate-buffered saline (PBS) to a 10% (w/v) dilution. After centrifugation at 3000*g* for 30 min at 4 °C, the supernatant was collected and aliquoted for storage at −70 °C until use. After polymerase chain reaction (PCR) detection, fifty-five samples were positive for HEV genotype 4, while one sample from a swine herd in the Zhejiang Province of China in the year 2011 was positive for the HEV genotype 3 (ZhJ-PJ050-3).

Total RNA was extracted from 10% (0.1 g/ml) fecal supernatant using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The entire genomic sequence of ZhJ-PJ050-3 was amplified using primer sets as described previously (Chobe *et al*., 2006). First strand complementary DNA (cDNA) was synthesized with the Transcriptor First Strand cDNA Synthesis Kit (Roche, Indianapolis, USA) and each external antisense primer. First round PCR was carried out using 4 μl of the synthesized cDNA and the external primer set with Ex Taq DNA polymerase (TaKaRa, Shiga, Japan). Nested PCR was then carried out with the respective internal primer set and 4 μl of the first PCR product. The products were cloned and sequenced to construct the full-length genome.

The 5' rapid amplification of cDNA ends (RACE) was carried out with SMARTer RACE 5'/3' Kit

‡ Corresponding author

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ORCID: Jiang-bing SHUAI, http://orcid.org/0000-0001-6540-1962

(Clontech, Madison, USA) as suggested by the manufacturer. Briefly, extracted RNA was treated with SMARTer II A Oligonucleotide (supplied with the kit). First-strand cDNA was synthesized by reverse transcription using 5'-CDS primer A and SMARTScribe Reverse Transcriptase (supplied with the kit). The cDNA was then amplified by two PCR rounds with SeqAmp DNA polymerase (Clonetech, Madison, USA) using Universal Primer A Mix and Nested Universal Primer A short (supplied with the kit) as forward primers, and genotype 3 HEV gene specific primers 191R and 127R as reverse primers. The resulting PCR products were purified, TA-cloned, and sequenced.

Amplification of the extreme 3' end was also carried out with SMARTer RACE 5'/3' Kit (Clontech, Madison, USA). cDNA was synthesized using 3' RACE CDS primer A, which has a poly(T) tract and SMARTScribe Reverse Transcriptase (supplied with the kit). The resultant cDNA was then amplified by two rounds of PCR using HEV gene specific primers 6373F and 6676F as forward primers, and Universal Primer A Mix and Universal Primer A short (supplied with the kit) as reverse primers, respectively. The PCR product was TA-cloned and sequenced.

Nucleotide (nt) and amino acid sequences were aligned using ClustalX 1.8. Genetic distances between pairs of HEV isolates were calculated with the Kimura two-parameter method and the phylogenetic tree was constructed with the neighbor-joining method using MEGA software version 5.0 (Tamura *et al*., 2011).

Twenty-two overlapping sequences were obtained to construct the full-length genome. The complete genome of ZhJ-PJ050-3 contained three recognized ORFs and was determined to be 7223 nt in length, excluding the poly(A) tail at the 3' terminus. The genome consisted of 5' UTR of 25 nt (1–25), ORF1 of 5109 nt (26–5134), ORF2 of 1983 nt (5169–7151), ORF3 of 342 nt (5158–5499), and 3' UTR of 72 nt (7152–7223), followed by a poly(A) tail of 23 nt. The sequence of ZhJ-PJ050-3 has been deposited with GenBank, accession No. KT633715.

The full-length genomic sequence of ZhJ-PJ050-3 shared a high nucleotide identity to isolates of genotype 3 (Table 1). Notably, the highest genomic nucleotide similarity of 87.9% was revealed between ZhJ-PJ050-3 and the reported genotype 3 HEV strain SAAS-JDY5 (GenBank, accession No. FJ527832) isolated from Shanghai, China (Si *et al*., 2009). The ORF1, ORF2, and ORF3 sequences of the two Chinese strains were 87.9%, 86.1%, and 93.6% similar, respectively. The nucleotide identity of ZhJ-PJ050-3 with genotype 1, genotype 2 (the Mexican strain), and genotype 4 isolates were 73.2%–73.9%, 72.9%, and 74.2%–75.5%, respectively. In total, 24 unique amino acid substitutions were identified as compared to other genotype 3 isolates as following positions (Table 2).

Table 1 Nucleotide and amino acid identity (%) of swine HEV isolate (ZhJ-PJ050-3) with complete genome sequences of 91 human and swine HEV isolates in GenBank*

Genotype	Full-length	ORF1	ORF2	ORF3
Genotype 1, *n*=12	73.2–73.9	70.5–71.2 (77.4–79.4)	79.1–80.2 (89.9–91.2)	83.6–86.0 (75.4–79.8)
Genotype 2, *n*=1	72.9	70.8 (78.9)	78.1 (89.4)	82.5 (75.4)
Genotype 4, *n*=23	74.2–75.5	72.3–73.3 (81.7–82.9)	79.4–81.4 (90.0–91.5)	84.4–87.1 (78.8–84.2)
Genotype 3, *n*=55				
China, *n*=1	87.9	87.9 (93.8)	86.1 (96.1)	93.6 (94.7)
Japan, *n*=30	79.2–87.0	77.5–87.1 (89.4–94.1)	82.9–87.3 (94.5–97.0)	91.7–95.3 (89.9–95.8)
Thailand, *n*=1	79.7	78.3 (90.2)	82.3 (94.5)	91.2 (92.9)
Mongolia, *n*=2	80.1–82.3	78.2–80.5 (89.6–91.8)	82.8–84.3 (94.4–94.7)	90.9–92.4 (88.5–92.0)
Korea, *n*=2	84.3–84.8	83.6–84.0 (92.6–92.7)	85.8–86.4 (94.7–95.2)	92.7–94.2 (94.7–96.5)
USA, *n*=3	84.7–85.2	83.5–85.7 (92.7–93.7)	83.0–87.7 (95.9–96.5)	93.0–95.3 (92.9–96.5)
Canada, *n*=3	84.5–85.4	83.3–84.7 (91.9–93.5)	86.0–86.5 (95.0–95.8)	91.8–94.2 (90.3–96.5)
Spain, *n*=5	78.6–79.9	78.3–84.7 (90.4–93.7)	82.5–86.4 (94.1–94.8)	90.9–92.1 (92.0–93.8)
France, *n*=2	78.7–82.0	80.5–83.6 (90.6–91.5)	82.4–83.2 (94.5–95.0)	92.1–92.4 (91.2–92.9)
Germany, *n*=3	79.4–82.7	77.6–81.2 (90.3–92.5)	82.4–85.6 (94.2–95.5)	90.9–93.0 (89.4–92.9)
UK, *n*=3	82.3–84.7	83.5–83.6 (92.6–93.1)	85.7–85.9 (95.0–95.9)	93.0–93.3 (93.8–94.7)

* Values in parenthesis indicate amino acid identity

Table 2 Unique amino acid substitutions identified in ZhJ-PJ050-3 as compared to other available genotype 3 isolates

Gene	Amino acid positions
ORF1	L57F, F95S, V294G, C384R, C434C, F548L, S592L, H918P, R975P, H1010L, L1123F, L1295F, L1316S, W1415R, M1515K, I153N, C1604G, L1663P, F1691L
ORF2	G134R, D188V, E222V, F635L
ORF3	L90H

Whole genome-based phylogenetic analysis confirmed the classification of swine HEV isolate ZhJ-PJ050-3 as genotype 3 (Fig. 1). According to the proposed reference sequences for HEV subtypes described by Smith *et al*. (2016), the phylogenetic tree revealed that ZhJ-PJ050-3 was clustered in subtype 3b together with most Japanese swine and human isolates, and was most closely related to the only Chinese isolate SAAS-JDY5 (FJ527832). Interestingly, HEV sequences in subtype 3b mainly originated from Japan, and nucleotide identities of ZhJ-PJ050-3 to Japanese strains in subtype 3b ranged between 85.2% and 87.0%, which were higher than the identities to genotype 3 strains from other areas (78.6%–85.4%).

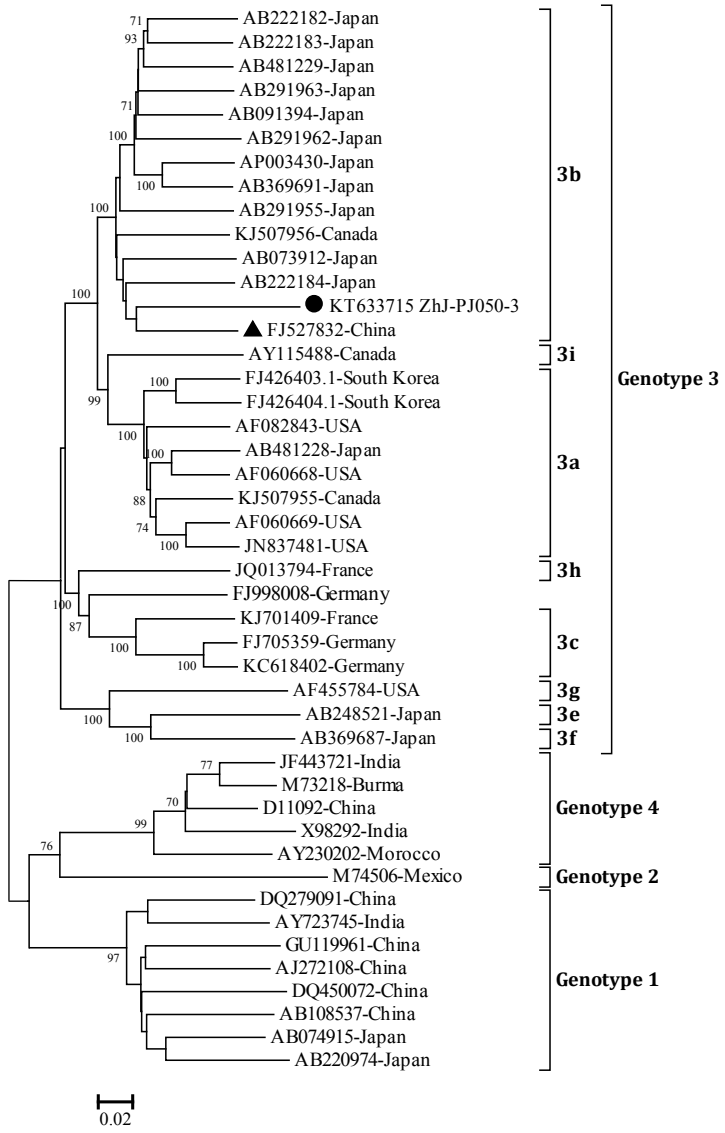

Fig. 1 Phylogenetic trees depicting genotypic status of ZhJ-PJ050-3 (indicated by ●) on the basis of full-length genome sequence of 47 HEV isolates

GenBank accession numbers for the sequences are shown at each branch. The reported genotype 3 HEV strain SAAS-JDY5 (FJ527832) isolated from Shanghai, China is indicated by ▲

The 25-nt 5' UTR of ZhJ-PJ050-3 was identified by 5' RACE. Alignment of the 5' UTR of available genotype 3 HEV strains revealed a very high level of conservation. Most genotype 3 isolates contained a 5' UTR of 25 nt in length, whereas a number of isolates had a 26-nt 5' UTR with an insertion of nucleotide G at the extreme 5' end.

ORF1 of ZhJ-PJ050-3 was comprised of 5109 nt (26–5134) potentially encoding a protein of 1702 amino acids. The size of ORF1 was identical to the Chinese isolate SAAS-JDY5 but shorter than most other genotype 3 swine and human isolates. One amino acid deletion of Leu residue at position 1048 was observed in the ZhJ-PJ050-3 ORF1 protein, which was the same as that of SAAS-JDY5 ORF1. ZhJ-PJ050-3 exhibited the highest level of ORF1 nucleotide identity with SAAS-JDY5 of 87.9%. Nucleotide and amino acid identities of ZhJ-PJ050-3 ORF1 with ORF1 of other human and swine genotype 3 isolates ranged from 77.5% to 87.1% and from 89.4% to 94.1%, respectively.

ORF2 of ZhJ-PJ050-3 consisted of 1983 nt (5169–7151). The encoded major capsid protein of 660 amino acids showed identical size to the ORF2 protein of other human and swine genotype 3 HEVs. Alignment of the ORF2 amino acid sequence of genotype 3 HEVs revealed that the N-terminal (residues 1–120) and C-terminal region (residues 477–654) containing the E2s domain (amino acids 459–606) with all identified neutralizing epitopes of the protein (Zhao *et al*., 2015) were more variable. Substitutions of residues unique to ZhJ-PJ050-3 were observed at positions 134 (G to R), 188 (D to V), 222 (E to V), and 635 (F to L). Nucleotide and amino acid identities of ZhJ-PJ050-3 ORF2 with the ORF2 sequences of other genotype 3 isolates ranged from 82.4% to 87.7% and from 94.1% to 97.0%, respectively. A partial ORF2 sequence of another two Chinese genotype 3 strains, swCNZJ304-06 isolated from swine in Zhejiang (EF781823, 304 nt) and Ech22 from human in Jiangsu (HM439285, 1681 nt), showed nucleotide identity of 88.7% and 86.6% to ZhJ-PJ050-3, respectively.

ORF3 of ZhJ-PJ050-3 with 342 nt (5158–5499) potentially encoded a protein of 113 amino acids. The amino acid identity of ZhJ-PJ050-3 ORF3 to ORF3 of SAAS-JDY5 was 94.7%. The ZhJ-PJ050-3 ORF3 exhibited highest amino acid similarities of 96.5% with an American (AF060668) and a Canadian isolate (AY115488). One unique substitution at amino acid position 90 (L to H) was confirmed.

The ORF2 of ZhJ-PJ050-3 terminated with a TAA codon, which was identical to other genotype 3 isolates. The following 3' UTR was comprised of 72 nt (7152–7223) excluding the poly(A) tail. Alignment of the 3' UTR of ZhJ-PJ050-3 with other available genotype 3 HEV sequences (n=55) revealed a considerable nucleotide diversity of the region. The length of 3' UTR of genotype 3 HEV isolated ranged from 67 to 73 nt. Comparing 3' UTR of ZhJ-PJ050-3 with SAAS-JDY5, the latter exhibited a deletion of nucleotide T at position 7186 nt and a two nucleotides addition (AC) at the very end of the 3' terminus preceding the poly(A) tail.

Hepatitis E is endemic and has become an important public health problem in many developing countries (Wenzel *et al*., 2011). Much research has provided sufficient evidence for zoonotic transmission of HEV (Meng, 2011). Genotypes 3 and 4 HEV were found both in human and in animal hosts such as pigs, horses, boars, deer, rabbits, and mongooses, among which swine is the most important animal reservoir (Meng, 2010; Wang *et al*., 2016b). Genotype 4 HEV is believed to be the predominant genotype and is prevalent in most regions of China, whereas genotype 3 HEV strains have been found in human populations and swine groups almost in eastern China including regions of Shanghai, Zhejiang, Jiangsu, and Anhui and were thought to be imported from Japan (Lu *et al*., 2013).

After the first detection of swine Chinese genotype 3 isolate in Shanghai (Ning *et al*., 2007), high HEV prevalence among Chinese swine herds was detected, indicating that genotype 3 HEV infections in swine had become more common in eastern China (Zhang *et al*., 2010a). Furthermore, Zhang *et al*. (2010b) revealed that genotype 3 HEV was also circulating in the human population in recent years in eastern China. These facts may provide a hint of zoonotic transmission of genotype 3 HEV strains in China. During 2010 and 2014, we detected six genotype 3 strains in fecal samples collected from swine herds in Zhejiang Province (Zhang *et al*., 2013), including ZhJ-PJ050-3. The nucleotide identity of the six strains ranged between 92.3% and 94.5% when compared with the 680 nt sequences of ORF2 (data

not shown). Moreover, another Zhejiang swine genotype 3 strain (EF187823, 304 nt) showed high partial ORF2 sequence similarity with ZhJ-PJ050-3. Thus, we postulate that genotype 3 HEVs circulating in Zhejiang Province are originally identical. The complete genome of ZhJ-PJ050-3 was then characterized in this study and phylogenetically analyzed with other genotype 3 strains from different regions in the world.

Recent study indicated that the genotype 3 HEV sequences reported from eastern China exhibited high genetic identity (Liu *et al*., 2012). Similarly, two HEV 3 strains from eastern China, ZhJ-PJ050-3 in this study and SAAS-JDY5 detected from Shanghai, shared a high nucleotide identity of 87.9% at whole genome level. ZhJ-PJ050-3 and SAAS-JDY5 were classified as subtype 3b and both displayed a close genetic relationship with most Japanese human and swine genotype 3 strains based on whole genome phylogenetic analysis. Moreover, nucleotide identities of ZhJ-PJ050-3 to Japanese strains were higher than the identities to genotype 3 strains from other areas. These findings may prove the speculation of importation of Chinese genotype 3 HEV from Japan. When ZhJ-PJ050-3 was compared with available sequences of human and swine genotype 3 isolates respectively, no distinct difference of nucleotide identities was observed (78.7%–87.0% vs. 78.6%–86.9%). Unique features were found associated with the ZhJ-PJ050-3 genome, including 24 amino acid substitutions in three ORFs. Further characterization is necessary to explore the meaning of genomic and biological features.

Compliance with ethics guidelines

Jiang-bing SHUAI, Lu-huan LI, Ai-yun LI, Yong-qiang HE, and Xiao-feng ZHANG declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要

题　目：基因 3 型猪戊型肝炎病毒浙江株全基因组序列分析

目　的：扩增浙江地区基因 3 型戊型肝炎病毒全基因组序列，分析其分子特征及与国内外 3 型戊型肝炎病毒进行比较。

创新点：首次分析报道浙江地区基因 3 型猪戊型肝炎病毒全序列。

方　法：利用套式 PCR 和末端快速扩增法对分离自浙江地区的戊型肝炎病毒进行分段扩增，经克隆测序后拼接全基因组。进一步利用生物信息学方法对基因组结构、开放阅读框及其基因型等进行分析，并与国内外戊型肝炎毒株进行比较分析。

结　论：浙江株戊型肝炎病毒（ZhJ-PJ050-3）基因组由 7223 个核苷酸组成，其中 3'非编码区含有 23 个碱基的 poly(A)尾。全基因组包含 ORF1、ORF2 和 ORF3 三个主要基因，长度分别为 5109、1983 和 342 bp。进化分析显示，ZhJ-PJ050-3 为基因 3 型，与国内唯一报道的上海株 3 型毒株以及多数日本毒株共属 3b 亚型。与国内外戊型肝炎序列比对发现，ZhJ-PJ050-3 基因组共有 24 个特有的点突变，且在 ORF1 中含有一个亮氨酸缺失。这些分子特征将为下一步的研究提供方向。

关键词：猪戊型肝炎病毒；全基因组序列；进化分析；基因 3 型